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Developmentally regulated loss of ubiquitin and ubiquitinated proteins during pollen maturation in maize

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ABSTRACT **Eukaryotic cells typically contain 0.2–1.0% of their total protein as the highly conserved protein ubiquitin, which exists both free and covalently attached to cellular proteins. The attachment of ubiquitin to cellular proteins occurs posttranslationally by a three-enzyme pathway and results in a peptide linkage of the C terminus of ubiquitin either to a lysyl ε-amino group of a substrate protein or to a lysyl ε-amino group of a previously linked ubiquitin molecule. The multiple conjugation of ubiquitin to substrate proteins via ubiquitin–ubiquitin linkages is thought to be necessary, but not sufficient, for recognition and degradation by a ubiquitin-dependent protease. In higher plant cells the steady-state level of ubiquitinated proteins is generally constant and can be readily detected in all somatic tissues. In contrast, we have found that a developmentally regulated loss of free ubiquitin and ubiquitinated proteins occurs during maize (*Zea mays* L.) pollen maturation. This dramatic loss of ubiquitin correlates temporally with commitment to the gametophytic developmental program. Northern blot analysis indicates that the loss of ubiquitin is not due to low levels of ubiquitin mRNA, suggesting that a posttranscriptional regulatory mechanism is responsible.**

The 76-amino acid protein ubiquitin, the three-enzyme pathway that covalently attaches ubiquitin to cellular proteins, and the enzymes that catabolize ubiquitinated proteins are found in all eukaryotic cells. In addition to short-lived ubiquitinated proteins apparently targeted for degradation, pulse labeling of cultured mammalian cells suggests a large pool of stable ubiquitinated proteins, whose cellular roles are not yet elucidated (1). The importance of protein modification by ubiquitin is underscored by the fact that ubiquitin modification of currently unknown protein substrates has been implicated in multiple fundamental cellular responses in eukaryotes, cell cycle progression, DNA repair, and in yeast, protein export, peroxisomal biogenesis, and sporulation (2–4). In mammalian cells, accelerated ubiquitin-mediated degradation of the nuclear protein p53 results in abnormal cell proliferation (5) and ubiquitinated histone 2B is preferentially found in actively transcribed chromatin of several animal species (6). In higher plants, the photoreceptor phytochrome that mediates red light photomorphogenic responses is ubiquitinated after photoconversion (7). Thus, ubiquitin conjugation plays multiple fundamental roles in cellular growth and physiology in diverse eukaryotes.

In higher plants, production of gametophytes (the 1*N* generation) is highly regulated and requires precise control of gene expression (8–10). The identification of numerous pollen-specific genes as well as effects of mitochondrial genome variation specifically on pollen development indicate that unique and specialized physiological processes occur in gametogenesis in higher plants (11–13). Maize male gametophyte production begins with differentiation of the haploid products of meiosis and proceeds through several distinct biochemical and morphological stages. This maturation program most notably includes a mitotic cell division, called microspore mitosis, that results in a marked asymmetric division of cellular contents. This division is thought to signal commitment of microspores to gametophytic development (14). Previous studies have shown that the RNA and protein populations of the pollen undergo a substantial alteration at this time (15, 16). Because of this dramatic change in protein composition, and because the pollen grain represents a unique developmental structure, we have investigated the steady-state level of free ubiquitin, ubiquitinated proteins, and ubiquitin mRNA in maturing pollen of different developmental stages.

MATERIALS AND METHODS

Isolation of Maize Microspores and Preparation of Protein Extracts. Male gametophytes were isolated from tassels of the inbred line Ky21 and fractionated into developmental stages by continuous sucrose gradients as described (16). The resultant developmentally staged cells have a high degree of viability (16). Mature pollen grains were collected from shedding tassels. Three different experiments using developing microspore fractions were analyzed. Protein extracts were prepared from all stages in one of two ways, which gave identical results for all immature stages and nearly identical results for mature pollen. In extraction procedure A, isolated staged gametophytes and pollen were resuspended in SDS sample buffer and immediately boiled. In extraction procedure B, isolated staged gametophytes and pollen were passed through a French Press (at 15,000 psi; 1 psi = 6.9 kPa) in buffer with protease inhibitors [10 mM Tris·HCl, pH 8/100 mM NaCl/5 mM EDTA/10% (vol/vol) glycerol/2.5 mM phenylmethylsulfonyl fluoride/10 mg of chymostatin, leupeptin, and pepstatin per ml]. Extraction procedure B gave slightly higher levels of ubiquitinated proteins in immunoblot assays for mature pollen. Protein content in the extracts was determined by the bicinchoninic acid dye binding assay according to the supplier's instructions (Pierce) relative to a bovine serum albumin standard.

Protein Gel and Immunoblot Analysis. Equivalent amounts of protein from male gametophytes at each of the stages were fractionated by SDS/PAGE, using either 7% or 15% acrylamide separating gels (17). One microgram of protein was used for visualization of total protein by silver staining (18). Anti-rabbit antibodies to denatured, cross-linked bovine ubiquitin (Sigma) were prepared and affinity purified as described (1, 19). Immunoblot analyses with 10 μg of protein were performed as described (20) and visualized with alkaline phosphatase-linked goat anti-rabbit secondary antibody and 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (20).

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RNA Isolation and RNA Gel Blot Analysis. Poly(A)$^+$ RNA was isolated from maize endosperm, nonvacuolated young microspores, starch-filled immature pollen, and mature pollen as described by Broadwater and Bedinger (21). Two micrograms of each poly(A)$^+$ RNA was fractionated in 1% agarose in 2.2 M formaldehyde buffer (22). RNA was blotted to Zeta-Probe GT membrane (Bio-Rad). An *Xba* I/*Hin*dIII DNA restriction fragment containing only ubiquitin coding sequences from the *Arabidopsis* polyubiquitin gene *UBQ3* (J.C., unpublished data) was purified by using Geneclean II (Bio 101) and radioactively labeled with ^{32}P (23). The blot was incubated with the probe in 0.25 M Na_2HPO_4, pH 7.2/7% SDS at 65°C, washed in 20 mM Na_2HPO_4, pH 7.2/1% SDS at 65°C, and exposed to x-ray film (Kodak) at −80°C with an intensifying screen (DuPont).

RESULTS

Protein extracts from developmentally staged microspores and pollen were analyzed for their content of ubiquitinated proteins (Fig. 1). Ubiquitin immunoblots revealed that the early stages of gametophyte development, when cells are uninucleate, contained readily detectable levels of ubiquitinated proteins typical of eukaryotic cells (lanes YM and MSV). This level declined slightly in fully vacuolated microspores at the point when microspore mitosis is initiated (lane LV). However, ubiquitinated proteins were barely detectable in protein extracts from bi- or tricellular gametophytes at the subsequent stage of starch-filled immature pollen and in protein extracts from mature pollen (lanes SF and MP). By densitometric analysis of immunoblots, we estimate that the reduction in levels of ubiquitinated proteins from young microspores to mature pollen is at least 50-fold.

In addition to the reduction in the level of ubiquitinated proteins that occurs during pollen maturation, the levels of free ubiquitin in partially starch-filled immature pollen (Fig. 2, lane PS) and mature pollen grains (lane MP) were >10-fold lower than that found in young microspores (lane YM). The ladder of abundant low molecular mass ubiquitinated proteins visible in Fig. 2 in addition to free ubiquitin correspond in size to ubiquitin covalently linked to additional ubiquitin moieties via Lys-48, forming free ubiquitin chains (25, 26). The kinetics of their disappearance is identical to that of higher molecular mass conjugates. Ubiquitin chains could represent intermediates in the attachment of ubiquitin to cellular proteins or represent breakdown products from previously attached ubiquitin (26, 27).

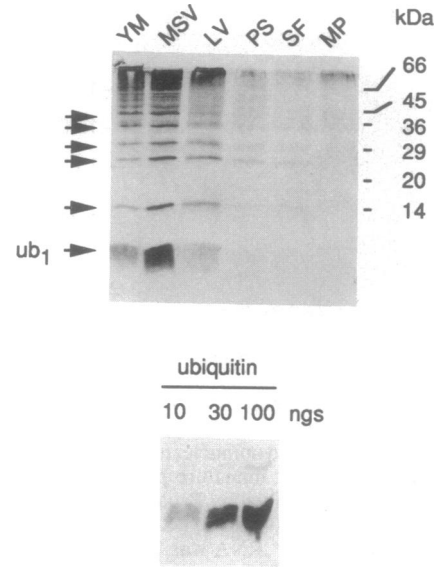

FIG. 2. Immunoblot analysis of free ubiquitin and low molecular mass ubiquitinated proteins during maize microspore development. (*Upper*) As in Fig. 1, except protein extracts were fractionated on a SDS/15% polyacrylamide gel. (*Lower*) Immunoblot titration of purified human ubiquitin (Sigma). No differences in immunoreactivity have been observed between human and higher plant ubiquitin (24). Abbreviations are as in Fig. 1.

One explanation for the absence of free ubiquitin in maturing pollen is that transcription of its genes is silenced in male gametophytes. Poly(A)$^+$ RNA was isolated from staged microspores and used in RNA gel blot analysis to determine whether ubiquitin mRNAs are present in microspores with little or no ubiquitin or ubiquitinated proteins. Previous studies have shown that maize seedling poly(A)$^+$ mRNA contains two major transcript size classes, the larger of which has been characterized. It consists of transcripts from two different genes, each containing seven ubiquitin coding regions (28, 29). Ubiquitin mRNA is present in all stages of pollen development (Fig. 3). The most abundant ubiquitin transcript size class visualized in maize endosperm and young microspore poly(A)$^+$ mRNA correspond in size to the

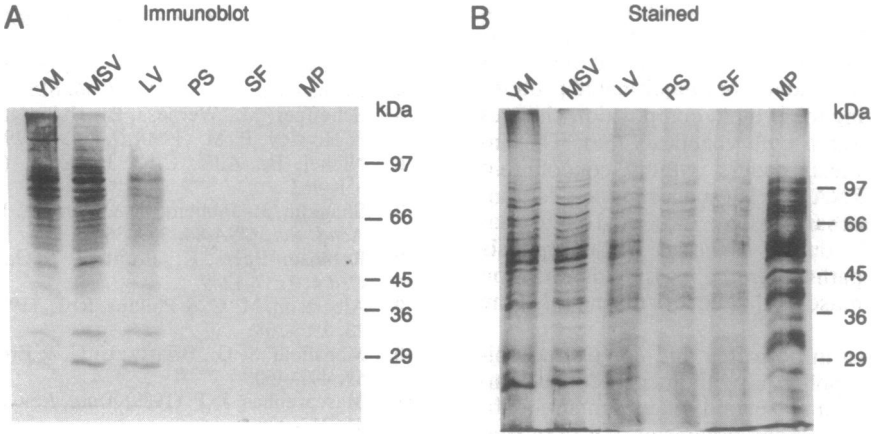

FIG. 1. Determination of the levels of ubiquitinated proteins during maize microspore development. Total microspore proteins were fractionated on a SDS/10% polyacrylamide gel. (*A*) Immunoblot of 10 μg of microspore proteins reacted with anti-ubiquitin antibodies as described. (*B*) Silver-stained polyacrylamide gel of 1 μg of the same extracts. Abbreviations indicate the developmental stage of the developing microspore and are as described and designated in ref. 14; YM, nonvacuolated young microspores; MSV, microspores with multiple small vacuoles; LV, microspores or young pollen with a single large vacuole; PS, partially starch-filled young pollen; SF, starch-filled young pollen; MP, mature pollen.

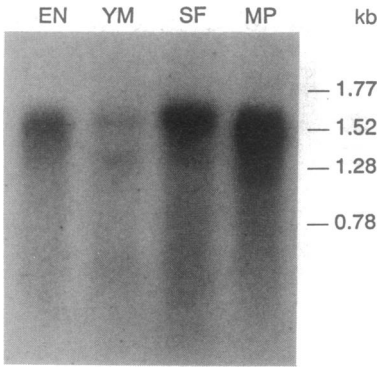

FIG. 3. RNA gel blot analysis of ubiquitin expression during pollen development. Poly(A)$^+$ RNA was isolated from maize endosperm (EN), nonvacuolated uninucleate young microspores (YM), starch-filled bi- or tricellular immature pollen (SF), and tricellular mature pollen (MP) as described by Broadwater and Bedinger (21). Positions of RNA markers (GIBCO/BRL) in kb are shown. Two micrograms of each poly(A)$^+$ RNA was fractionated in 1% agarose as described and hybridized to an *Arabidopsis* ubiquitin coding region sequence.

major ubiquitin transcript size classes in seedling RNA (data not shown). RNA gel blot analysis demonstrated that the steady-state level of ubiquitin mRNAs did not decrease during pollen maturation but rather increased somewhat (Fig. 3). This is in marked contrast to the changes in levels of ubiquitin and ubiquitinated proteins (Figs. 1 and 2). Pollen at the starch-filling stage is translationally active (11, 14). Therefore, posttranscriptional regulatory mechanisms specific for ubiquitin mRNAs may explain the reduction of ubiquitin protein in maturing pollen.

DISCUSSION

The developmentally regulated loss of ubiquitin and ubiquitinated proteins during pollen maturation is distinct from previous reports of developmentally regulated changes in the ubiquitin pathway. Others have reported tissue-specific expression of a component or several components of the ubiquitin conjugation/deconjugation pathway: for example, the neuronal-specific expression of a ubiquitin-dependent deconjugating enzyme, PGP 9.5 (30), the human sperm-specific expression of an isozyme of ubiquitin activating enzyme (E1) (31, 32), and changes in conjugating enzyme (E2) composition during erythrocyte maturation (33–35). Developmentally regulated increases in ubiquitin gene expression have also been documented. Increases in the rate of ubiquitin synthesis and in the levels of ubiquitinated proteins have been observed during the protein composition changes that occur during sea urchin embryogenesis (36). This increase in ubiquitin synthesis occurs without concomitant changes in ubiquitin mRNA levels, suggesting a posttranscriptional regulatory mechanism (36). Increased accumulation of ubiquitin mRNAs during *Manduca* metamorphosis implicates the ubiquitin pathway in the massive degradation of larval intersegmental muscles that occurs during this time (37, 38).

The dramatic reduction in both free and conjugated ubiquitin found in maturing pollen is without precedent compared to other tissues within maize and other plants (unpublished data), and indeed in other organisms (19, 25), and invites speculation as to its functional significance. The roles of ubiquitin within the cell are still not completely defined but generally include targeting proteins for proteolysis, some aspect of chromatin structure and/or gene expression, and stress response (3). It is possible that the lack of the ubiquitination pathway could simply reflect the transient nature of mature male gametophytes in higher plants. In keeping with this idea is the finding that, although there is a heat shock response in higher plant somatic and embryonic tissues, pollen is distinctive in the lack of induction of heat shock protein mRNA levels and synthesis of heat shock proteins upon heat treatment (39). The timing of the loss of both ubiquitin and the heat shock response correlates with the event of microspore mitosis, which is thought to mark commitment to the gametophytic developmental program (16, 40). Pollen, which survives only for a short burst of pollen tube growth after release from the anthers, may not require tight regulation of protein turnover or of protein stabilization and therefore has eliminated both systems from its physiological repertoire. The demonstrated presence of ubiquitin mRNAs in mature pollen indicates that such an elimination would occur via a posttranscriptional mechanism.

Another possible reason for the loss of ubiquitin during pollen maturation is that ubiquitin recognition of aberrant protein structures (2) and protein aggregates (41) may be deleterious within pollen. As pollen matures, it accumulates storage substances and proteins necessary for the rapid growth of a pollen tube that delivers the gametes to the embryo sac for fertilization. Some of the proteins accumulating to high levels at this time are cytoskeletal proteins such as actin (16) and tubulin (39). These normally filamentous proteins are stored in a nonfilamentous form, ready to be rapidly polymerized upon tube germination. In addition, a rather drastic dehydration occurs during pollen maturation, such that the water content of mature maize pollen is only 40–58% by weight (42). This contrasts with the 80–90% water content of younger stages of male gametophytes and of somatic plant cells. Such a dramatic difference in water content might have consequences in terms of protein–protein interactions, and perhaps even in protein conformation. It is possible that the presence of an active ubiquitin pathway in such an environment might lead to recognition of these altered conformations, wrongly targeting proteins for degradation.

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